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In vivo oxidizability of LDL in type 2 diabetic patients in good and poor glycemic control

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Abstract

We aimed to determine if increased non-enzymatic glycosylation of the LDL was sufficient to increase the susceptibility to in vivo oxidation of the LDL particles. Twenty-two type 2 diabetic patients (11 males and 11 females) were included in this study. They were enrolled on the basis of good [glycated hemoglobin (HbA_{1c}) < 7%] and poor glycemic control [(HbA_{1c}) > 8%]. LDL were isolated by sequential ultracentrifugation and analyzed by capillary electrophoresis (CE) for diene conjugate content and for electronegativity. The glyc-LDL levels were increased in all diabetic type 2 patients, peaking in the diabetic subjects in poor diabetic control (17.3 ± 8.07%). The LDL content of diene conjugates was similar between the two groups (6.65 ± 0.77% for the patients with good glycemic control versus 6.88 ± 0.74% for those with poor glycemic control; $P = 0.49$) as was the electrophoretic mobility ($(-1.14544 \pm 0.089) \times 10^{-4}$ cm^2/(V s) for the patients with good glycemic control and $(-1.13666 \pm 0.073) \times 10^{-4}$ cm^2/(V s) for those with poor glycemic control; $P = 0.80$).

The susceptibility to in vivo oxidation of LDL from type 2 diabetic patients in poor glycemic control did not differ from that of well-controlled diabetic patients. LDL glycosylation was not able to increase the oxidizability of LDL in the diabetic patients with poor glycemic control.

Keywords: Diabetes; Glycosylation; LDL oxidation; Diene conjugates; Capillary electrophoresis

1. Introduction

The increased atherogenic risk associated with diabetes is accentuated by the numerous differences described between lipoproteins from diabetic and non-diabetic individuals [1,2]. Because of hyperglycemia most plasma apolipoproteins from diabetic subjects become glycosylated and impaired in their biological function [3]. Glycosylation is a process that increases the negative charge of LDL [4]. Modifications that increase the net negative charge may have important metabolic consequences and enhance LDL atherogenicity [5,6]. Glycated LDL (glyc-LDL) is catabolized more slowly than normal LDL [7,8], and is degraded by the scavenger pathway promoting foam cell formation [9]. The advanced glycation end-products (AGE) process is one of the main pathogenic mechanisms linked to the development of diabetic complications [10]. AGE may arise via several mechanisms. In non-oxidative pathway, AGE can be produced by reaction of reducing sugars with protein amino groups throughout the cascade of Maillard reaction. The oxidative pathway of AGE formation involves the participation of reactive oxygen species producing glyco-oxidative products [10]. Increased oxidative-stress probably contributes to the increased susceptibility to oxidation [11]. An increase in AGE accumulation precedes the histological evidence of diabetic microvascular damage. Type 2 diabetes complications primarily affect the vascular system, leading to diabetic microangiopathy and to an accelerated development of atherosclerosis [12].

The electrophoretic mobility is one of the more reliable indicators of LDL modification. Conventionally, changes in the electrophoretic mobility of LDL particles were determined in agarose gel using a barbital buffer, or in capillary electrophoresis (CE) [13].

It has been reported that in type 2 diabetes the majority of LDLs are small and dense, especially at high triglyceride levels. Small dense LDL particles are triglyceride-enriched lighter particles that reside in slow turnover metabolic pool [14]. It was found that small dense LDLs are more

Abbreviations: glyc-LDL, glycosylated LDL

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susceptible to oxidation [15]. To determine the susceptibility of LDL to in vivo oxidation we have applied the measure of the amount of baseline diene conjugation (BDC) in LDL [16], which is a clinically applicable method to estimate in vivo LDL oxidation. Strong correlation between the titer of autoantibodies against oxidized LDL and results of high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) studies indicated that BDC-LDL was a specific measure of oxidized LDL in vivo [16,17].

The present study examined the proportion of circulating glycated LDL in type 2 diabetic patients and the LDL susceptibility to oxidation through the precise measurement of the electrophoretic mobility by capillary electrophoresis and the UV absorption at 234 nm that results from the formation of conjugated dienes in constituent polyenoic fatty acids.

2. Subjects and methods

2.1. Study subjects

Twenty-two type 2 diabetic patients (11 males and 11 females) were included in this study. They were recruited from the diabetes clinic at the San Giovanni Battista Hospital in Turin (Italy). They were enrolled on the basis of good [glycated hemoglobin(HbA_{1c}) < 7%] and poor glycemic control [(HbA_{1c}) > 8%]. Diabetes was defined according to the National Diabetes Data Group criteria [18]. For the diabetic subjects in good glycemic control mean age was 67 ± 9 years, body mass index (BMI) was 29 ± 5 kg/m^2, and known diabetes duration was 8 ± 8 years. For the diabetic subjects in poor glycemic control mean age was 63 ± 12 years, body mass index (BMI) was 31 ± 3 kg/m^2, and known diabetes duration was 12 ± 5 years. Anthropometric characteristics, glycemic control parameters, and lipid profiles of type 2 diabetic patients are shown in Table 1.

All the patients were on hypoglycemic oral agents and none of the patients was taking drugs or vitamins, or had any disease known to influence lipoprotein metabolism.

Table 1
Anthropometric characteristics, glycemic control parameters, and lipid profiles of type 2 diabetic patients

	Diabetic subjects with good glycemic control	Diabetic subjects with poor glycemic control
Sex (M/F)	6/5	6/5
Age (year)	67 ± 9	63 ± 12
Diabetes duration (year)	8 ± 8	12 ± 5
BMI (kg/m^2)	29 ± 5	31 ± 3
HbA_{1c} (%)	6.4 ± 0.58**	10.2 ± 0.9
Glucose (mg/dl)	136 ± 43**	195 ± 53
Cholesterol (mg/dl)	215 ± 48	201 ± 48
Triglycerides (mg/dl)	146 ± 44	166 ± 55
LDL cholesterol (mg/dl)	135 ± 49	124 ± 43
HDL cholesterol (mg/dl)	51 ± 15	47 ± 11

** $P < 0.01$.

Eleven healthy subjects matched for sex, age, and lipid profile were recruited as a control group.

Informed consent was obtained from all the enrolled subjects and the reported investigations were carried out in accordance with the principle of the Declaration of Helsinki [19].

2.2. Biochemical analyses

Glucose was determined by a standardized automatized enzymatic method (glucose oxidase) (Alfa Wassermann, Milan, Italy) adapted to a Schimadzu CL 7000 autoanalyzer. Cholesterol and triglyceride concentrations were determined from plasma and lipoprotein fraction by enzymatic methods (Alfa Wassermann, Milan, Italy) in a Schimadzu CL7000 autoanalyzer.

2.3. Isolation of LDL

Venous blood was collected from all participants into EDTA-containing Vacutainer tubes, and plasma was separated by low-speed centrifugation for 15 min at 4 °C. LDL was isolated by preparative sequential ultracentrifugation [20] in a Beckman L8-70M ultracentrifuge (Beckman Instruments Inc.). Plasma was adjusted to $d = 1.019$ g/ml with KBr solution and ultracentrifuged at 42 000 rpm (121 000 g) for 20 h at 18 °C in a Beckman 70.1 Rotor. After centrifugation, the very low density lipoprotein (VLDL) fraction was removed by the tube slicing and the infranate was adjusted to $d = 1.063$ g/ml and ultracentrifuged at 46 000 rpm (148 000 g) for 21 h at 18 °C. To avoid oxidative modification of lipoproteins 1 mmol/l EDTA was added to all KBr solutions in the two sequential ultracentrifugations. The fraction containing LDL was removed by tube slicing and dialyzed through Sephadex G-25 columns (Pharmacia) against buffers used in the subsequent experiment.

2.4. Chromatographic assays

HbA_{1c}: HbA_{1c} was routinely determined by standardized affinity high performance liquid chromatography (Bio Rad, Italy). The reference range in our laboratory is 4.0–5.8%.

Glyc-LDL: Glyc-LDL percentage was evaluated by affinity chromatography using *m*-amino-phenyl-boronic acid coupled with agarose (Sigma) as described [21]. Five milliliters of the resin was packed in a glass column at a flow of 1 ml/min. The column was connected to a low pressure chromatography system. The column was equilibrated with binding buffer (250 mmol/l ammonium acetate, 50 mmol/l $MgCl_2$, 500 mmol/l NaCl, 3 mmol/l NaN_3, and 0.1% Tween-20, pH 8.5 at 20 °C. An aliquot of LDL was dialyzed against binding buffer by gel filtration chromatography (G25M; Pharmacia) and about 400 μg of LDL were injected into the column. Non-glyc-LDL was eluted in the unbound fraction with 20 ml binding buffer at a flow rate of

1 ml/min. The glycated LDL fraction was eluted with elution buffer (200 mmol/l sorbitol, 500 mmol/l NaCl, 50 mmol/l Na_2-EDTA, 100 mmol/l Tris, and 0.1% Tween-20, pH 8.5. Two peaks of LDL, corresponding to non-glyc-LDL and glyc-LDL were detected at 254 nm and their peak area were integrated.

2.5. Capillary electrophoresis

Capillary electrophoresis was performed with a Beckman P/ACE 5510 System fitted with a diode array detector. An untreated fused silica capillary tube with a 75 μm i.d. was used. The total length of the capillary tube was 57 cm (50 cm to the detector). The capillary was operated at 20 °C. An applied voltage of 350 V/cm was used in all CE separations. Capillary electrophoresis was performed as described by Stock and Miller [13]. The cathode and anode electrolytes and the capillary run buffer were 40 mM methylglucamine-Tricine, pH 9.0. LDL samples were injected by low pressure for 4 s. Dimethylformamide was injected as an electroendosmotic flow (EOF) marker for 1 s. A voltage of 24 kV was applied ramping over 0.8 min. Migration of LDL particle was monitored at 200 and 234 nm. The amount of conjugated dienes is obtained from the percentage of the height of LDL peak at 234 nm related to the height of LDL peak at 200 nm.

2.6. Calculation of electrophoretic mobility

The electrophoretic mobility of LDL (μ) was calculated using the following equation [22]: $\mu = L_c L_d / U(1/t_{sample} - 1/t_{EOF})$ where L_c is the total length of the capillary (cm), L_d the length of the capillary from injector to detector (cm), U the applied voltage (V), t_{sample} the migration time of LDL in seconds, and t_{EOF} is the migration time of the EOF marker in seconds.

2.7. Statistical analysis

Data were expressed as mean ± S.D. unless otherwise indicated. Student's *t*-test was used to compare groups when variables were normally distributed. Pearson's correlation coefficients were used to describe relationships between variables. In all cases, *P* of a value less than 0.05 was considered statistically significant.

3. Results

The diabetic patients in good glycemic control had a HbA_{1c} levels of 6.4 ± 0.58%, and those with poor diabetic control had a HbA_{1c} value of 10.2 ± 0.9% ($P = 0.000$). The fasting glucose concentrations were 136 ± 43 and 195 ± 53 mg/dl, respectively, ($P = 0.009$) (Table 1).

As expected, type 2 diabetic patients with poor glycemic control presented increased proportion (17.3 ± 8.07%) of glycated LDL compared with type 2 diabetic subjects with good glycemic control (9.73 ± 4.32%; $P = 0.01$).

Table 2
Glyc-LDL proportion and parameters of LDL susceptibility to oxidation

	Diabetic subjects with good glycemic control	Diabetic subjects with poor glycemic control
Glyc-LDL (%)	9.73 ± 4.32*	17.3 ± 8.07
Electrophoretic mobility ($\times 10^{-4}$ cm^2/(V s))	−1.14544 ± 0.089	−1.13666 ± 0.073
Diene conjugates (%)	6.65 ± 0.77	6.88 ± 0.74

* $P < 0.05$.

The LDL electrophoretic mobility mean for the diabetic group was $(-1.14015 \pm 0.08) \times 10^{-4}$ cm^2/(V s). The electrophoretic mobility of LDL between the two diabetic subgroups did not vary ($(-1.14544 \pm 0.089) \times 10^{-4}$ cm^2/(V s) for the patients with good glycemic control and $(-1.13666 \pm 0.073) \times 10^{-4}$ cm^2/(V s) for those with poor glycemic control; $P = 0.80$) (Table 2). In both diabetic groups, LDL electrophoretic mobility was significantly higher than that found in the control group ($P < 0.05$ control versus both groups).

The contents in diene conjugates of LDL is 6.76 ± 0.75% in the whole group and it is higher than the level found in the control group (5.9 ± 0.3; $P = 0.001$). No significant difference was observed between the diabetics in good (6.65 ± 0.77%) and poor glycemic control (6.88 ± 0.74%; $P = 0.49$) (Table 2).

Table 3 shows the Pearson's correlation coefficients between the concentration of glycated LDL and those of HbA_{1c}, glucose, LDL electrophoretic mobility and content in diene conjugates, and the correlation coefficients between diene conjugates and LDL electrophoretic mobility in the different group of diabetic subjects. Glyc-LDL correlated positively with HbA_{1c} in the whole group of diabetic subjects ($r = 0.65$; $P = 0.0009$). Within the diabetic group this

Table 3
Pearson's correlation coefficients between serum glycated LDL and HbA_{1c}, plasma glucose levels, LDL electrophoretic mobility and LDL diene conjugates content and Pearson's correlation coefficients between LDL diene conjugates and electrophoretic mobility

	All diabetic subjects	Diabetic subjects with good glycemic control	Diabetic subjects with poor glycemic control
N	22	11	11
Glyc-LDL vs.			
HbA_{1c}	0.65**	−0.16	0.74**
FPG	0.58**	0.18	0.54
Electrophoretic mobility	0.64**	0.49	0.74**
Diene conjugates vs.			
Electrophoretic mobility	−0.68**	−0.68*	−0.71*

* $P < 0.05$.
** $P < 0.01$.

correlation is kept only in the subjects with poor glycemic control ($r = 0.74$; $P = 0.009$).

Glyc-LDL correlated positively with glucose in the whole group of diabetic subjects ($r = 0.58$; $P = 0.004$), but this correlation faded away when the statistical analyses was performed on the two separated groups.

Glyc-LDL correlated positively with electrophoretic mobility in the whole group of diabetic subjects ($r = 0.64$; $P = 0.001$), and in the subjects with poor glycemic control ($r = 0.74$; $P = 0.008$).

Glyc-LDL correlated negatively with the amount of diene content in the whole group of diabetic subjects ($r = -0.66$; $P < 0.0006$), and in the patients with HbA_{1c} level <7% ($r = -0.76$; $P = 0.006$). The diabetic patients with poor diabetic control did not present a significant correlation between glycated LDL and diene conjugated content.

The content in diene conjugates of LDL showed a good correlation with the electrophoretic mobility both in the whole group ($r = -0.68$; $P = 0.0005$) and in the subgroups with good ($r = -0.68$; $P = 0.02$) or poor glycemic control ($r = -0.71$; $P = 0.01$). Although this correlation is expressed with a negative sign, it has in reality a positive meaning because the electrophoretic mobility of LDL is usually expressed with a negative value.

4. Discussion

Our study was designed to quantify the fraction of glycosylated LDL in type 2 diabetic patients and to determine if the susceptibility of LDL to oxidation is altered in type 2 diabetic patients in poor glycemic control. We aimed to determine if increased non-enzymatic glycosylation of the LDL was sufficient to increase the susceptibility of the LDL particles in vivo.

As expected, the glyc-LDL levels were increased in all diabetic type 2 patients when compared to those of control subjects ($4 \pm 0.8\%$) and the highest concentration was found in patients in poor glycemic control. Our results of glycated LDL are far higher than those published by other authors [23,24]. Moreover, to be sure that no non-specific substances were bound to the resin, we added Tween-20 as a detergent according to Fless and coworkers [21]. The absorbance of the peaks was measured at 254 nm and not at 280 nm as others reported [23], and this wavelength allows greater sensitivity. Others authors [24] have used an immunoassay method to quantify the percentage of glycated LDL. So far, no standard methods for glycated LDL exist and the heterogeneity of the employed laboratory methods could explain these discrepancies.

The susceptibility to in vivo oxidation of LDL from type 2 diabetic patients in poor glycemic control did not differ from that of well-controlled diabetes. The LDL content of diene conjugates was similar between the two groups, as was the electrophoretic mobility was. The increase in absorbance measured at 234 nm parallels the formation of conjugated dienes in constituent fatty acids, the production of thiobarbituric acid-reacting substances, and the accumulation of lipid hydroperoxides and cholesterol peroxidation products [25–27], whereas the measure of the electrophoretic mobility of LDL is considered an indicator of lipoprotein modifications [28]. An increased electronegativity of LDL is the result not only of the oxidative damage [25] and the extent of cholesterol oxidation [29], but also of glycosylation. Both oxidation and non-enzymatic glycosylation share an increase in the electronegativity of LDL as a common characteristic [23]. The lack of difference in electrophoretic mobility of LDL observed between the two groups suggests that the glycosylation of LDL has reached the saturation levels. LDL glycosylation, which reflects the modification of lipoprotein, accounts for the increased electrophoretic mobility of diabetic LDL compared to that of LDL isolated from a control group. LDL glycosylation was not able to further increase the oxidizability of LDL in the diabetic patients in poor glycemic control. In agreement with that, the negative correlation found between glycated LDL and diene conjugates explains that glycosylation can offer lipoproteins more resistance to the further oxidation process as other authors have suggested [30]. We suppose that the similar oxidizability observed in LDL of the two diabetic groups is due to the early phases of the LDL oxidation which are delayed by glucose as long as the vitamin E content is preserved. LDL vitamins E content was not different in the diabetic subjects with good or poor glycemic control (data not shown). However, we expected the rate of LDL oxidation to speed up once LDL vitamin E has been consumed as other authors have reported [31].

The high circulating glucose level accounts for the non-enzymatic glycosylation of LDL as proved by the significant correlation of glyc-LDL with glucose and HbA_{1c}. In these patients LDL are clearly modified and these modifications are highlighted by the correlation of the LDL content in diene conjugates with the LDL electrophoretic mobility existing both in the whole group ($r = -0.68$; $P = 0.0005$) and in the subgroups with good ($r = -0.68$; $P = 0.02$) or poor glycemic control ($r = -0.71$; $P = 0.01$). As expressed above, despite the negative sign, the correlation has in reality a positive meaning since the electrophoretic mobility of LDL is usually expressed with a negative value [22].

In addition to the saturation theory, we do not exclude that the capillary electrophoresis be able to detect modified LDL due to glycation irrespective of the number of glucose molecules attached to lipoproteins.

Higher electric charge and enhanced degree of glycation are physiochemical and biochemical parameters differentiating lipoproteins in type 2 diabetic patients from lipoproteins in non-diabetic individuals. These lipoprotein modifications can contribute to the increased risk of coronary atherosclerosis (CHD) associated with diabetes [1,2]. These observations led us to agree with other authors [24] who supposed that glyc-LDL could be used as a sensitive index of short-to-long term glycemic control in diabetes

[32,33]. Glyc-LDL measurement could also simultaneous provide an index of the relative atherogenicity of circulating LDL, especially as glyc-LDL has a higher content of diene conjugates and higher electrophoretic mobility than normal subjects.

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